A COMPARISON OF HORIZONTAL VERSUS VERTICAL MIXING PROCEDURES AND PLASTIC VERSUS GLASS PETRI DISHES FOR ENUMERATING BACTERIA IN RAW MILK[1]

C. N. HUHTANEN[2], A. R. BRAZIS[3], W. L. ARLEDGE[4], E. W. COOK[5], C. B. DONNELLY[6], S. E. FERRELL[7], R. E. GINN[8], L. LEMBKE[8], D. J. PUSCH[9], E. BREDVOLD[9], H. E. RANDOLPH[10], E. L. SING[11], AND D. I. THOMPSON[12]

ABSTRACT

A comparison was made of a horizontal versus vertical shaking procedure for raw-milk dilutions used in the Standard Plate Count for the enumeration of milk bacteria. No significant differences ($P<0.01$) were found. Glass and plastic petri dishes were also compared in a like manner and no significant differences could be detected.

The method recommended by *Standard Methods (1)* for mixing milk dilutions consists of 25 vertical cycles of 1 ft length to be completed in a 7 sec period. This method was shown to give higher bacterial counts than two gentler inversion methods *(2)*. The vertical movements, however, are rather tiring when handling a large number of milk samples. A horizontal mixing procedure would be far easier to perform for extended time intervals. The experiments embodied in this report were set up to determine whether differences exist between vertical and horizontal mixing procedures.

A preliminary study was made by one of the laboratories cooperating in this study which indicated that glass petri dishes gave higher plate counts than plastic dishes. This was a disturbing report and indicated that a definitive comparative study of these two types of petri dishes was needed for the standard plate count since both are now in widespread use. The second part of these experiments, carried on in conjunction with the vertical and horizontal mixing study, was therefore intended to answer the question of glass versus plastic petri dish equivalence for plating raw-milk samples.

MATERIALS AND METHODS

Eight laboratories participated in these studies. Six laboratories assayed six raw-milk specimens each and two laboratories assayed twelve each for a total of sixty milk samples tested. Two of the laboratories, in addition, reported the results for two separate analysts on the same milk specimens. Samples examined included those from farm bulk tanks and commingled samples from storage tanks at the manufacturing facility. The procedures used for assaying bacterial counts were those in *Standard Methods (1)* with the single exception of the horizontal mixing procedure for the dilution bottles. The plastic petri dishes used by the different investigators were purchased with no attempt made to use the product of a single manufacturer. Duplicate petri dishes were poured for each test condition. The total plate counts were usually calculated from petri dishes showing between 30 and 300 colonies; however, in all instances counts were made from the same dilution in order to avoid introducing a possible dilution variance in addition to the method variances. The statistical analysis of the results was performed in a manner similar to that of the previous report *(2)* using a 1% level of significance. Plate counts were transformed to logarithms in order to normalize the statistical distributions. Tests for reproducibility between the two mixing methods, the two types of petri dishes, and between laboratories were performed by calculating average variances for each and testing by the F ratio using the 1% level of significance.

[1]A contribution from the Subcommittee for the Examination of Milk and Milk Products, Applied Laboratory Methods Committee, International Association of Milk, Food, and Environmental Sanitarians, Inc.

[2]Eastern Utilization Research and Development Division, U.S. Dept. of Agriculture, Philadelphia, Pa. 19118 (Chairman of the Subcommittee).

[3]Bureau of Foods, Pesticides, and Product Safety; Food and Drug Administration; 1090 Tusculum Ave.; Cincinnati, Ohio 45226 (Chairman, Applied Laboratory Methods Committee).

[4]Southeast Milk Sales Association, POB 1099, Bristol, Va. 24201.

[5]Quality Control Laboratory, Industrial Highway, Southampton, Pa. 18966.

[6]Bureau of Foods, Pesticides, and Product Safety; Food and Drug Administration; 1090 Tusculum Ave.; Cincinnati, Ohio 45226.

[7]Central States Cooperative, 355 West Second St., Superior, Nebr. 68978.

[8]Quality Control Laboratory, 2274 Como Ave. West, St. Paul, Minn. 55108.

[9]Division of Laboratory Services, Minnesota State Dept. of Agriculture, St. Paul, Minn. 55101.

[10]Dept. of Animal Science, Texas A and M University, College Station, Texas 77843.

[11]Moseley Laboratories, 3862 East Washington St., Indianapolis, Ind. 46201.

[12]State Laboratory of Hygiene, 437 Henry Mall, Madison, Wis. 53706.

TABLE 1. EFFECT OF MIXING METHODS AND TYPE OF PETRI DISH ON PLATE COUNTS (IN THOUSANDS PER ML)

Laboratory number	Milk sample no.	Vertical mix				Horizontal mix			
		Plastic dish		Glass dish		Plastic dish		Glass dish	
1	1	14.6	16.0	16.2	15.8	16.5	16.6	16.4	16.3
	2	5.1	5.3	5.4	5.6	6.1	5.8	5.9	5.6
	3	19.8	20.0	20.4	19.4	21.0	21.3	21.4	21.8
	4	8.3	9.0	10.7	10.0	11.5	10.0	12.2	11.4
	5	20.8	21.0	21.8	22.0	19.1	19.5	19.6	19.9
	6	25.0	24.6	26.8	25.7	27.4	27.2	30.0	29.8
	Average	15.8		16.6		16.8		17.5	
2	7	6.8	5.7	6.1	6.5	6.2	5.9	6.6	5.0
	8	17:5	17.3	21.0	18.6	17.9	17.5	19.3	16.6
	9	5.0	6.4	5.8	6.2	6.7	5.5	5.7	6.2
	10	14.8	13.8	13.8	16.8	12.5	18.9	16.3	19.5
	11	8.6	9.0	7.9	10.3	9.9	8.0	8.1	7.1
	12	13.9	14.9	13.3	11.7	12.5	12.6	13.8	14.3
	Average	11.1		11.5		11.2		11.5	
3	13	7.2	6.6	7.1	6.6	6.4	6.0	7.0	7.0
Analyst	14	92	85	95	84	75	79	86	78
A	15	59	57	75	69	55	51	52	67
	16	48	40	43	42	46	43	42	42
	17	88	78	88	77	69	72	68	79
	18	32	35	28	34	20	24	29	27
	Average	52.3		54.1		45.5		48.7	
3	13	6.8	6.9	8.7	9.1	6.6	8.6	7.1	6.9
Analyst	14	96	87	90	82	101	90	93	89
B	15	78	85	97	85	82	79	66	60
	16	44	44	44	38	41	41	42	44
	17	101	97	115	107	86	89	100	115
	18	26	24	35	38	25	25	39	35
	Average	58.2		62.4		56.2		58.1	
4	19	28.2	26.5	29.0	28.1	24.9	26.3	25.9	28.8
	20	211	206	223	227	202	205	228	230
	21	47	47	48	49	43	45	49	42
	22	6.9	7.0	6.8	7.2	6.2	7.2	7.1	8.2
	23	8.7	8.1	9.7	10.4	9.5	8.5	9.2	10.2
	24	168	142	145	145	138	130	143	140
	Average	75.5		77.4		70.5		76.8	
5	25	13.4	15.1	14.7	14.5	18.3	14.2	15.3	14.6
	26	16.9	19.2	19.5	18.9	20.8	20.0	17.7	18.1
	27	4.1	5.1	5.5	5.8	4.0	5.1	6.5	6.2
	28	13.8	13.0	17.2	17.1	16.8	13.1	12.7	15.1
	29	92	106	97	80	89	104	99	76
	30	60	37	62	44	47	34	78	68
5	31	12.3	12.4	11.2	12.7	11.8	10.8	12.3	12.6
	32	21.6	18.4	19.4	21.3	16.7	18.5	13.4	15.6
	33	6.6	6.4	6.3	5.1	6.4	4.6	5.9	5.8
	34	15.2	18.0	18.4	14.9	16.0	19.2	18.3	16.9
	35	9.7	10.8	9.5	8.3	10.7	13.0	8.7	8.7
	36	7.3	7.7	4.1	4.5	5.5	6.8	4.9	6.5
	Average	22.6		22.2		21.9		23.2	
6	37	10.2	10.5	11.1	8.4	8.9	8.1	8.3	10.1
	38	14.7	15.0	11.7	14.6	17.2	14.3	13.6	14.1
	39	8.0	4.8	6.4	8.0	8.1	7.2	7.3	6.3
	40	3.8	3.3	4.0	3.9	4.4	3.9	4.0	3.1
	41	3.3	3.2	3.9	3.5	3.8	4.8	2.3	2.8
	42	20.7	22.2	19.8	20.1	20.9	22.6	20.9	21.5
	43	100	120	93	102	95	103	112	95
	44	5.0	4.6	4.7	6.0	4.4	5.4	5.0	4.6

		45	13.9	13.4	10.8	12.9	13.6	13.4	11.1	13.0
		46	16.8	16.4	15.7	15.5	15.8	16.8	14.1	14.4
		47	28.4	22.9	25.6	24.2	23.9	24.2	26.5	25.7
		48	8.4	8.6	8.0	9.1	9.0	8.3	6.9	8.9
	Average		19.9		18.4		19.0		18.8	
	7	49	296	347	327	311	334	287	345	324
		50	49	52	56	49	54	51	51	42
		51	65	67	90	74	79	75	78	86
		52	328	313	407	395	234	259	206	237
		53	66	46	63	59	52	65	60	60
		54	90	75	92	104	87	92	101	94
	Average		149.5		168.9		139.1		140.3	
Analyst A	8	55	27.2	25.8	22.9	23.7	27.4	24.8	26.8	25.8
		56	12.3	13.5	14.1	12.1	16.1	17.2	16.1	16.9
		57	21.8	24.9	24.2	21.6	21.7	22.1	22.2	19.4
		58	35	38	43	40	39	44	46	44
		59	35	38	30	39	34	34	34	37
		60	12.9	14.3	14.3	14.8	14.3	13.5	15.5	13.9
	Average		24.9		25.0		25.7		26.5	
Analyst B	8	55	25.1	26.3	26.2	25.1	23.3	22.6	23.8	22.9
		56	14.6	12.7	16.3	13.8	14.9	13.8	13.7	14.4
		57	24.7	23.2	23.6	24.6	23.0	22.5	22.6	23.0
		58	29	37	28	39	39	31	33	37
		59	29.7	27.6	28.0	29.9	27.5	28.3	27.4	26.6
		60	12.9	14.1	12.8	13.9	13.3	12.0	12.9	14.1
	Average		23.1		23.4		22.6		22.6	
Over-All Average			41.3		43.4		39.1		40.5	

Results and Discussion

Average plate counts

The duplicate and average plate counts for 60 samples of milk are shown in Table 1. Two analysts assayed the same milk samples in Laboratory No. 3 and also in Laboratory No. 8. These results were further summarized by combining the counts from mixing methods and type of petri dish (Table 2). The results of the averaged plate counts in this table suggested that the vertical mix gave higher plate count than the horizontal. Actually 4 of the 10 analysts (from Laboratory No. 1, 2, 5, and 8A) found higher counts with the horizontal method than with the vertical, although the overall average of the vertical method was 5.9% higher than the horizontal. Only one analyst, however, (Laboratory No. 6) found the plastic dishes to give higher counts than the glass. The glass dishes gave an average of 4.2% more colonies than the plastic.

The question of which method or variation is "superior" is subject to some individual interpretation. It would, of course, be very desirable to be able to enumerate each individual bacterial cell in a sample. This, however, is seldom practical. A method giving higher counts would generally be considered superior to one giving lower counts. An important—perhaps the most important—criterion, however is that of increased sensitivity or reproducibility of one method over another. This aspect of these analyses will be considered later in this report.

Statistical analyses

The apparent superiority of the averaged values for the vertical mixing method over the horizontal and the glass petri dishes over the plastic was analyzed further using standard analysis-of-variance procedures. The results are summarized in Table 3. The milk samples (line A) were, as expected, highly significantly different in the variations in average plate counts of bacteria. Part of this difference was accounted for as an investigator's variance (line B). The previous report *(2)* had failed to detect this source of variation at the same level of significance. Most of the variation of the milk samples was, however, caused by the samples themselves (line C) rather than by the different laboratories.

Main effects. The analysis-of-variance of treatment effects (line D) failed to show any significant differences among the four different treatments at the 1% level of significance or, for that matter, at any level of significance. The conclusion must therefore be that no differences were demonstrated between the vertical and horizontal shaking methods or between the glass and plastic petri dishes.

Interactions. The interactions resulting from these

TABLE 2. AVERAGE PLATE COUNTS BY LABORATORIES COMPARING TWO TYPFS OF PETRI DISHES AND TWO MIXING METHODS (PLATE COUNTS IN THOUSANDS PER ML)

Laboratory number	Number samples assayed	Mixing method		Type of petri dish	
		Vertical	Horizontal	Plastic	Glass
1	6	16.2	17.2	16.3	17.1
2	6	11.3	11.4	11.2	11.5
3A	6	53.2	47.1	48.9	51.4
3B	6*	60.2	57.1	57.1	60.2
4	6	76.5	73.6	73.0	77.1
5	12	22.4	22.6	22.2	22.7
6	12	19.2	18.9	19.5	18.6
7	6	159.2	139.7	144.3	154.6
8A	6	24.9	26.1	25.2	25.7
8B	6*	23.2	22.6	22.8	23.0
All Laboratories	60	42.3	39.8	40.2	41.9

*Analysts 3B and 8B assayed the same six milk samples of their counterparts, 3A and 8A.

TABLE 3. ANALYSIS-OF-VARIANCE SUMMARY OF RAW-MILK BACTERIAL COUNTS[a]

Line	Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	Significant ($P < 0.01$)
A	Milk Samples	59	104.8136037	1.7765018	522	Yes
B	Investigators	7	45.8576504	6.5510929	5.78	Yes
C	Samples within investigators	52	58.9559533	1.1337683	980	Yes
D	Treatments	3	0.0079833	0.0025510	0.78	No
E	Treatments X Samples Interactions	177	0.6736908	0.0038062	3.29	Yes
F	Investigators X Treatments	21	0.1431172	0.0068151	2.00	Yes[b]
G	Investigators X Plastic vs Glass	7	0.0433172	0.0061882	1.82	No
H	Investigators X Horiz. vs Vert.	7	0.0558996	0.0079856	2.35	No[c]
I	Residual	7	0.0439004	0.0062715	1.84	No
J	Treatments X Samples Within Investigators	156	0.5305736	0.0034011	2.94	Yes
K	Error (between duplicate plates)	240	0.2775849	0.0011566		
	Total	479	105.7728627			

[a]The following ratios were used for obtaining the F values: lines A/J, B/C, C/K, D/J, E/K, F/J, G/J, H/J, I/J, J/K.
[b]F ratio was on the borderline of significance at the 1% level.
[c]F ratio was, however, significant at the 5% level.

experiments are also summarized in Table 3 in lines E-J. There was a significant interaction (line E) at the 1% level of significance. It appeared that most of this interaction occurred as a result of the lack of uniform response of the various milk samples to the four treatments. This could have resulted from differences in the numbers or types of bacteria present. The glass petri dishes, for example, might have caused the agar to solidify more rapidly thus decreasing destruction of thermally-sensitive psychrophiles. There was a slight indication of investigator bias or preference for either the horizontal or vertical mixing procedure (line H) but this was only apparent at a lower level of significance (5%) than that

TABLE 4. AVERAGE VARIANCE ESTIMATES OF BACTERIAL PLATE COUNTS AMONG TREATMENTS[a]

Laboratory number	Vertical mix		Horizontal mix		Average variance estimate
	Plastic petri dish	Glass petri dish	Plastic petri dish	Glass petri dish	
1	0.0002642	0.0001710	0.0003584	0.0001258	0.002299
2	0.0016340	0.0023372	0.0040608	0.0024754	0.0026268
3A	0.0011134	0.0013131	0.0008199	0.0015932	0.0012099
3B	0.0003983	0.0009682	0.0013516	0.0007110	0.0007677
4	0.0005972	0.0001546	0.0006847	0.0010522	0.0006222
5	0.0031861	0.0023510	0.0043954	0.0016714	0.0029010
6	0.0033103	0.0023298	0.0015023	0.0022892	0.0023829
7	0.0030690	0.0012578	0.0014480	0.0011947	0.0017399
8A	0.0008364	0.0017724	0.0005120	0.006756	0.0009491
8B	0.0015438	0.0023915	0.0011225	0.0004114	0.0013673
Average variance estimate	0.0015953	0.0015137	0.0016256	0.0012200	

[a]These variance estimates (standard deviations squared) were calculated from the pooled variance estimates between duplicate plates using logarithmically transformed counts.

decided upon for the experiment.

Reproducibility among methods

The logarithmically transformed counts between duplicate plates were translated into estimates of variance (squares of standard deviations), pooled, and averaged over laboratories and methods. The final average variance estimates are given in Table 4. The reproducibility among treatments was not significantly different using the Cochran test. There also were no significant differences among the laboratories in the precision of the results obtained when an F test of the average variances was performed. All variances were well within the variance of log plate counts suggested by Donnelly et al. *(3, 4)* for reproducibility among investigators (maximum suggested variance was 0.012 in log units).

REFERENCES

1. American Public Health Association. 1967. Standard methods for the examination of dairy products, 12th ed. American Public Health Association, Inc., New York, N. Y.

2. Huhtanen, C. N., A. R. Brazis, W. L. Arledge, E. W. Cook, C. B. Donnelly, R. E. Ginn, J. N. Murphy, H. E. Randolph, E. L. Sing, and D. I. Thompson. 1970. Effect of dilution bottle mixing methods on plate counts of raw-milk bacteria. J. Milk Food Technol. 33:269-373.

3. Donnelly, C. B., E. K. Harris, L. A. Black, and K. H. Lewis. 1960. Statistical analysis of standard plate counts of milk samples split with state laboratories. J. Milk Food Technol. 23:315-319.

4. Donnelly, C. B., J. T. Peeler, and L. A. Black. 1966. Evaluation of state central milk laboratories by statistical analyses of standard plate counts. J. Milk Food Technol. 29:19-24.

THE CYCLAMATE STORY

(Continued from Page 399)

bladder cancer may be induced under certain conditions by the amino acid tryptophan (a constituent of most proteins) and has also been causally related to cigarette smoking; that there is serious question whether the sensitivities to cyclamate of the bladders of humans and rats are similar in the face of evidence that they are dissimilar for a number of compounds; and that selecting as the culprit one of two compounds in a mixture (the other partner being cholesterol in the implantation studies, saccharin in the feeding tests) seems arbitrary at best. Much criticism was leveled at the Delaney clause which most toxicologists, as well as HEW Secretary Finch recognize as a dogmatic law precluding any scope for the application of reasoned scientific judgment, such as consideration of dose-response relationships. Despite these criticisms and the fact that the full report of the FDRL data has not yet appeared, the use of cyclamates has been withdrawn or restricted in every country where it had been permitted.